



# Characterization of the Effects of IVIg on CNS Inflammation Leading to Non-Classical Disease in a Relapsing Remitting Model of EAE

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Characterization of the Effects of IVIg on CNS Inflammation Leading to Non-Classical  
Disease in a Relapsing Remitting Model of EAE

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A Thesis in the Field of Biology

for the Degree of Master of Liberal Arts in Extension Studies

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## Abstract

Intravenous Immunoglobulin (IVIg) is a complex therapeutic used in the treatment of multiple autoimmune and inflammatory diseases. The mechanism of action of IVIg is pleiotropic; there is increasing evidence that modulation of migration through the expression or function of selectins such as very late antigen-4 (VLA-4) may be central to therapeutic activity.

The impetus for the present studies arose from an unexpected and novel observation that was made while investigating IVIg treatment in a relapsing remitting (RR) model of experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis (MS). Specifically, treatment with IVIg led to a shift from classical ascending paralysis to a non-classical ataxic disease. Others have demonstrated that this change in phenotype reflects a shift in localization of inflammation from the spinal cord to the brain and brain stem in EAE. In line with recent reports showing that shifts from classical to non-classical disease phenotype is mediated by shifts in the relative abundance of T helper cells (Th1 and Th17 cells), we hypothesized our observation may reflect an effect of IVIg on VLA-4 mediated migration leading to differential localization of Th1 and Th17 cells to the CNS during inflammation.

In the process of investigating this hypothesis three key observations were made:

- 1) There was a decrease in B cells in the CNS with IVIg treatment; we hypothesize the impacted B cells were regulatory B cells, based on the timing and context of their development.

2) An increase in CD8<sup>+</sup> cells was observed in the anterior CNS associated with increased inflammation and demyelination; this population is hypothesized to be the effector population involved in the observed disease phenotype

3) We found evidence in the periphery suggesting IVIg has Th1 modulating activity that is overtly similar to anti-VLA-4 treatment in EAE reported previously by others.

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## Chapter I

### Introduction

Intravenous Immunoglobulin (IVIg) is increasingly being used as therapy for many inflammatory diseases that are either refractory to normal standard of care or that lack effective treatments to begin with or where further immune suppression is counter indicated (Dalakas 2004). The immuno-therapeutic activities of IVIg have been shown to be pleiotropic and the contribution of any given aspect of these activities in a particular application is not completely understood. In an effort to better understand the activities of IVIg, we addressed the consequences of treatment in experimental autoimmune encephalomyelitis (EAE), the animal model for multiple sclerosis (MS). This led to an unexpected observation that potentially implicated IVIg's role in modulating cellular migration leading to a change in disease phenotype from classical ascending paralysis to the non-classical ataxic form of the disease. Based on what is currently known about IVIg's activities in regards to modulation of adhesion molecules and our initial observations made in EAE, we hypothesized that IVIg was modulating certain subsets of T cells (Th1 & Th17) from normally migrating to the central nervous system in EAE via modulation of migratory integrins such as VLA-4 and LFA-1.

The purpose of the present study is to investigate this hypothesis. The specific aims include:

- 1) To demonstrate whether the T cell populations we hypothesize to be affected by IVIg are modulated in the CNS.
- 2) To show whether in full or part, the differences in these populations are due to effects of IVIg in the periphery during T cell priming.
- 3) If proven correct about the modulation of these T cell populations, whether this effect was due to the specific integrins we hypothesized to be, namely VLA-4 and LFA-1.

### Autoimmune Disease and Inflammation

The normally functioning immune system specifically recognizes and eliminates foreign agents thereby protecting the host against infections. During maturation of the immune system, adaptive immune cells that react against self-tissues are eliminated providing an immune system that is tolerant to “self”. It is when this tolerance breaks down that autoimmunity can arise. Autoimmune diseases affect approximately 5–8% of the population in the United States and are the third most common category of diseases in industrialized countries following cardiovascular diseases and cancer. Because many autoimmune diseases start at a relatively young age and continue chronically throughout life, they have a disproportionate effect on public health with an estimated annual cost of over 100 billion dollars in the United States alone (Fairweather 2001).

As the understanding of the root causes and cell types behind various autoimmune conditions has grown, more specific therapies have been developed tailored to the particular disease or syndrome. It is now understood that the adaptive immune responses mediated by lymphocytes (i.e. B cells and T cells) play a crucial role in the initiation and exacerbation of many autoimmune conditions. (Ziemssen and Schrempf

2007). One such condition that has received a great deal of attention in terms of research and funding, and has provided many insights into the interactions of various components of the immune system during autoimmune diseases is Multiple Sclerosis (MS).

### Multiple Sclerosis and Experimental Autoimmune Encephalomyelitis

MS is an autoimmune disease of the CNS that affects 0.1 % of the population in temperate latitudes. It is characterized by a wide range of neuropathology from headaches and hallucinations to deficits in coordination to paralysis. It is a lifelong disease with no cure at the moment. The majority of patients diagnosed with MS endure repeated cycles of relapse and remittance (RR) of symptoms. Over time a majority of patients experience a secondary progressive form of the disease (SP) with increasing severity of symptoms and shortened periods of remission. In the most severe form of MS, primary progressive MS (PP), patients deteriorate rapidly without remission and often succumb to the disease in a couple of years. The causes of MS are unknown but likely involve a combination of genetic predisposition and environmental factors (Ziemssen and Schrempf 2007).

EAE is an animal model of MS that has proven to be very valuable in the understanding of ongoing human disease. Indeed, both EAE and MS share many similar biological underpinnings from clinical and histological in disease phenotype, cell populations represented in the disease process as well as cytokine/chemokine repertoires (Zamvil and Steinman 1990, Steinman and Zamvil 2006). Importantly, EAE has been very useful in deepening our understanding of the mechanisms involved in T cell

trafficking and in the pre-clinical development of the anti-VLA-4 monoclonal antibody therapy, Natalizumab (Tysabri®) (Yednock, Cannon et al. 1992, Polman, O'Connor et al. 2006).

EAE can either be induced by direct immunization (active induction), or by adoptive transfer of CD4<sup>+</sup> T cells (passive induction). In the actively induced disease, immunologic tolerance is broken by immunizing animals with neuro-antigens, including proteolipid peptide/protein (PLP), myelin oligodendrocyte glycopeptide/protein (MOG), or myelin basic peptide/protein (MBP), in the presence of a pro-inflammatory adjuvant such as complete Freund's adjuvant (CFA). These models address early events in antigen presentation and lymphocyte activation/polarization, as well as later events such as cell migration from secondary lymphoid tissue (Beeton, Garcia et al. 2007, Bittner, Afzali et al. 2014). In passive transfer models, encephalitogenic T cells are generated from donor animals then re-stimulated *ex vivo* to become fully differentiated and activated effector cells. These cells are then adoptively transferred to recipient animals where they migrate to the CNS tissues and induce inflammation similarly to the later events of active induction models (Voskuhl 1996).

Although EAE and MS share many similarities, they also exhibit some important differences, in particular regarding the location of lesions. EAE is classically characterized by ascending paralysis, affecting primarily the spinal cord, while MS lesions are disseminated throughout the CNS. In EAE, complete paralysis occurs only in severe cases and inflammation is then found throughout the spinal cord, up to the cervical region. When brain inflammation is observed in EAE models, it is often associated with “non-classical” or “ataxic” disease phenotype (Tsunoda, Kuang et al. 2000, Wensky, Furtado et al. 2005, Lees, Golumbek et al. 2008). This is in contrast with a majority of

MS patients, where lesions are mostly observed in the brain and spinal cord inflammation is seen in a subset of patients (Simmons, Pierson et al. 2013, Simmons, Liggitt et al. 2014). Although the key events leading to MS and the underlying reasons for the discrepancies with EAE are not fully understood, in actively induced EAE the activation of various neuro-antigen specific leukocytes in the periphery and their trafficking to the CNS is a key step.

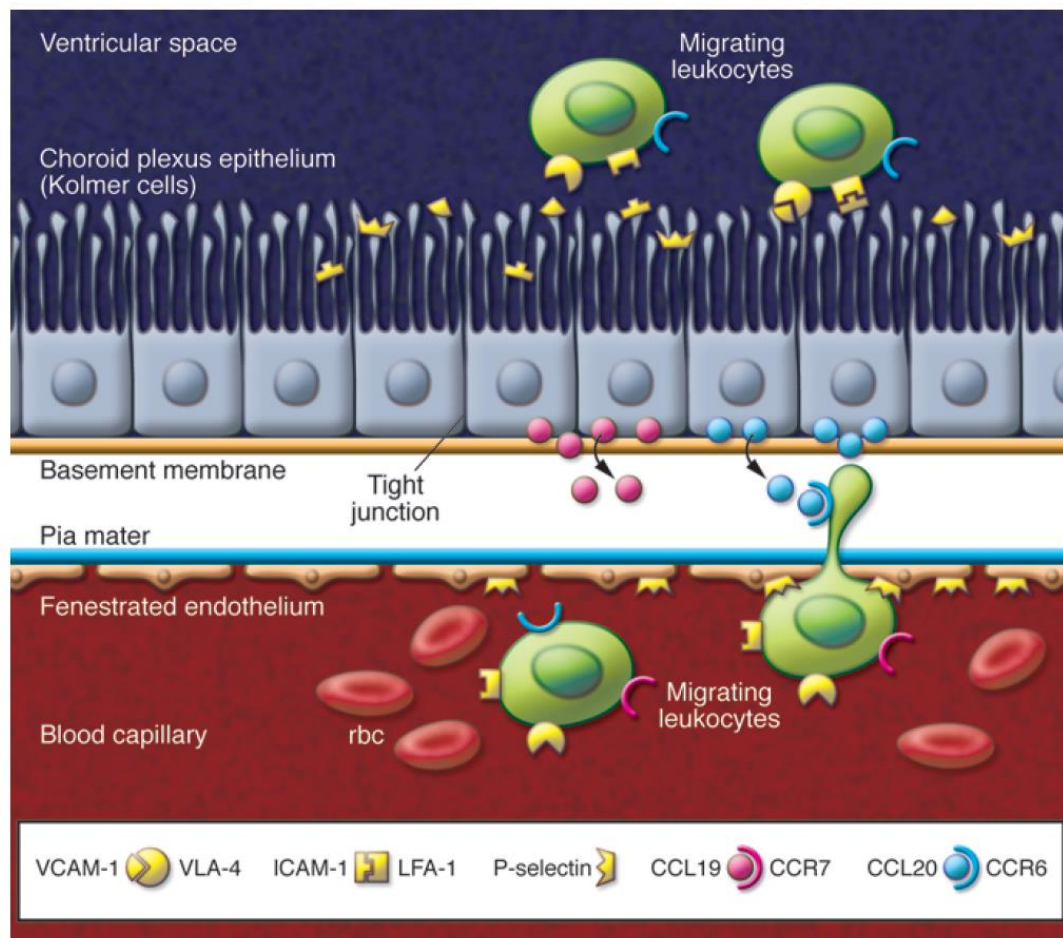
#### Activation, Polarization and Trafficking of T helper (Th) cells

Activation of naïve T cells and their differentiation into various Th subsets is the crucial event in the adaptive immune response. The local cytokine milieu and complex signaling events at the immune synapse between the antigen presenting cells (APC) and the antigen-specific Th cells determine the fate of these cells. Several varieties of Th cells are now recognized. For example, Th1 cells are associated with the cytokines IL-12 and IFN $\gamma$ , while Th17 cells primarily secrete IL-17 and arise from the influence of IL-6 and TGF- $\beta$ . Th2 cells primarily secrete IL-4 and IL-13 and regulatory T cells (Tregs) are induced from exposure to TGF- $\beta$  or IL-10 and are vital to quelling inflammatory responses (Luckheeram, Zhou et al. 2012). In autoimmunity, Th1 and Th17 cells often work in concert to stimulate and direct effector functions of macrophages and neutrophils. In addition to secreting different cytokines and chemokines that induce effector functions in different leukocyte populations, these activated Th cells also express different sets of cellular adhesion molecules (CAMs) allowing them to migrate to various tissues during immune surveillance and inflammation. Of particular relevance to these studies are the CAMs known as integrins (Golias, Tsoutsi et al. 2007).



Integrins are a large family of calcium dependent transmembrane receptors that can bind to other cells or extracellular matrix (ECM). They are heterodimers containing an alpha and beta subunit, which can combine to form at least 24 different integrins. They are normally expressed in a low affinity form but become activated when the cell encounters activating signals and able to bind their ligands with high affinity. IgSFs, like intercellular adhesion molecules (ICAMs) and vascular cell adhesion molecule-1 (VCAM-1), are constitutively expressed on endothelial cells and serve as ligands for the various integrins expressed on leukocytes. Selectins (P-, L- and E-) are a family of heterophilic CAMs that bind fucosylated carbohydrates on other CAMs or ECM. One of the best characterized ligands for selectins is P-selectin glycoprotein ligand-1 (PSGL-1), which is found on leukocytes. PSGL-1 binds to all three selectins but has the highest affinity for P-selectin (Golias, Tsoutsi et al. 2007).

During inflammatory processes, activated immune cells that pass through post capillary venules encounter various CAMs known as selectins (L-, P-and E-selectin) that have been upregulated on the endothelial cells as a result of ongoing inflammation. (See Figure 1) The interaction of selectins on these leukocytes with their ligands causes the cells to slow down. Subsequently, low affinity integrins on the surface of leukocytes (e.g. very late antigen (VLA)-4 and lymphocyte function-associated antigen (LFA)-1) bind their ligands on the endothelium (VCAM-1 and ICAM-1 respectively), slowing the cells down further and leading to cell arrest on the endothelial surface followed by extravasation across the endothelial layer into the perivascular space (Golias, Tsoutsi et al. 2007, Wilson, Weninger et al. 2010).



**Figure 1. Illustration of migration, adhesion and extravasation:** Process of migration, adhesion and extravasation of leukocytes in the CNS and relevant molecules (Adapted from Wilson et al.) (Wilson, Weninger et al. 2010).

Positive results observed in animal models and human clinical trials validated the inhibition of integrins as a therapeutic approach, with the monoclonal antibodies anti-VLA-4 (Natalizumab; Tysabri®) and anti-LFA-1 (Efalizumab; Raptiva®) being approved for Multiple Sclerosis (MS) and psoriasis, respectively (Gonzalez-Amaro, Mittelbrunn et al. 2005). However as with many immunosuppressive drugs, treatment was associated with an increased risk of various kinds of infections. One especially dire side effect for both drugs is progressive multifocal leukoencephalopathy (PML), a lethal form of

meningitis with no treatment, which is brought about by the reactivation of latent John Cunningham virus (JCV). For Raptiva® the cost/benefit ratio was too high and the drug was pulled from the market in 2009. For Tysabri®, it has been determined that the cost to benefit ratio is low enough to justify the risk with stringent screening and monitoring (Gonzalez-Amaro, Mittelbrunn et al. 2005, Kawamoto, Nakahashi et al. 2012, Romme Christensen, Ratzer et al. 2014). Intravenous Immunoglobulin (IVIg) has also been demonstrated to modulate VLA-4 activity, but in contrast to the dire side effects of these highly targeted immune therapies, IVIg has been shown to be well tolerated with few side effects.

### Intravenous Immunoglobulin (IVIg)

IVIg is a complex mixture of human IgG antibodies derived from the pooled serum of many thousands of healthy donors. IVIg is approved in the United States for the treatment of Chronic Inflammatory Demyelinating Polyneuropathy (CIPD), Immune Thrombocytopenic Purpura (ITP), Primary Immunodeficiency states, Secondary Immunodeficiency in Chronic Lymphocytic Leukemia and Kawasaki's disease. It is a well-tolerated first line therapy that is also used "off-label" for the treatment of a variety of other inflammatory, autoimmune and neuromuscular disorders (Jolles, Sewell et al. 2005, Leong, Stachnik et al. 2008). Initial trials in MS showed some promise of efficacy, but double blind placebo controlled studies failed to reach clinical significance in both RRMS and SPMS (Dalakas 2004, Hommes, Sorensen et al. 2004, Fazekas, Lublin et al. 2008). The reasons for these failures are yet unclear.

The effects of IVIg are pleiotropic and there is evidence for its activity on a broad range of biologies, including the modulation of pathogenic antibodies, inhibition of complement activation and interception of membrane attack complexes, modulation of Fc receptors on myeloid cells, down-regulation of inflammatory cytokines, suppression of T cell function and interference with antigen recognition, amongst others (Dalakas 2004, Jolles, Sewell et al. 2005).

A growing body of evidence suggests that one of the primary mechanisms of action for IVIg consists of modulating the expression of adhesion molecules, therefore affecting the migration of pathogenic leukocytes to sites of inflammation. *In vitro* rolling assays across coated flow chambers and cultured endothelium, as well as intravital microscopy studies, have shown modulations of VLA-4/VCAM-1, LFA-1/ICAM-1 and PSGL-1/P-selectin interactions leading to decreased adhesion of leukocytes when treated with physiological concentrations of IVIg. Interestingly, IVIg treatment seems to decrease the surface expression of some of these molecules, including PSGL-1 and LFA-1 (Lapointe, Herx et al. 2004, Gill, Doig et al. 2005) (Anthony, Wermeling et al. 2008) (Dole, Bergmeier et al. 2007, Urzainqui, Martinez del Hoyo et al. 2007, Huang, Sun et al. 2010).

In addition to modulating migration across inflamed blood vessels, there is evidence that IVIg also affects trafficking of lymphocytes from secondary lymphoid tissues. It was observed that IVIg treatment led to a down regulation of sphingosine-1 phosphate receptor (S1PR) in CD4 cells in the peripheral lymph nodes of EAE mice, leading to sequestration and decreased CNS infiltration. S1PR is a G-coupled protein receptor required for many functions including chemotaxis and migration, which is successfully targeted by Fingolimod (FTY720, Gilenya®) in RRMS. Taken together, leukocyte migration and the direct modulation of associated adhesion molecules seem to be

important targets for the activity of IVIg, although this does not rule out possible direct effects on CD4 T cell differentiation and proliferation (Othy, Hegde et al. 2013). In particular these observations have implications for IVIg's effects on various cell populations that differentially express adhesion molecules such as Th1 and Th17 T cells.

### Th1 vs Th17 in EAE and MS

Recent work has shed light on the similarities and differences between EAE and MS. MS, similar to most autoimmune diseases, is a highly heterogeneous condition. Most patients present with symptoms indicative of brain inflammation first; however some present with spinal cord inflammation only (optico-spinal MS) as with many EAE models. Interestingly, lesion samples from brains and spines of MS patients show similar characteristics to those seen in the brains and spines of mice. Namely, active lesions found in the brains of MS patients are highly positive for IL-17 secreting cells that had infiltrated the neural parenchyma (Tzartos, Friese et al. 2008, Pierson, Simmons et al. 2012, Simmons, Pierson et al. 2013).

It was originally believed that the major players in EAE and MS were CD4<sup>+</sup> T cells mainly comprised of a Th1 phenotype, characterized by the secretion of high levels of the cytokine IFN $\gamma$ . It is now recognized in the case of EAE and MS that both Th1 and Th17 cells play a pathogenic role in the disease process although the exact contribution of each subset is not yet fully understood (Jager, Dardalhon et al. 2009, Sie, Korn et al. 2014). The understanding of the mechanisms by which these various Th polarized CD4 cells may differ in the way they migrate to the CNS and begin or exacerbate the inflammatory

process has lately been of growing interest. In particular, recent studies have shown that T cell differentiation (Th1 or Th17) is associated with a differential expression of integrins, resulting in differing abilities to migrate to various tissues (Jager, Dardalhon et al. 2009, Glatigny, Duhon et al. 2011, Rothhammer, Heink et al. 2011, Schneider-Hohendorf, Rossaint et al. 2014). These studies have begun to shed light on the relationship between the Th1 and Th17 autoimmune responses in EAE and the differential role cellular adhesion molecules play in these Th subsets, including VLA-4/PSGL-1 on Th1 cells and LFA-1 on Th17 cells.

In models induced by immunization with the MOG<sub>35-55</sub> neuroantigenic peptide, animals show an acute monophasic disease that resembles PPMS. Interestingly, adoptive transfer of CD4<sup>+</sup> T cells differentiated *ex vivo* either as Th1 or Th17 in the MOG<sub>35-55</sub> model leads to two distinct forms of disease. Th1 cells induce a classical pattern of disease with ascending paralysis and lesions primarily located in the spinal cord with parenchymal infiltration of IFN $\gamma$ <sup>+</sup> T cells. In contrast, when cells are cultured to produce a predominantly Th17 phenotype, a non-classical disease phenotype is observed characterized by severe ataxia and lesions located in the cerebellum and brain stem with IL-17<sup>+</sup> T cells infiltrating the neural parenchyma. In addition, blocking VLA-4, which is predominantly expressed on Th1 cells, produced a similar change in disease from classical to atypical phenotype in both active and passive EAE models (Rothhammer, Heink et al. 2011). Furthermore, non-classical disease was inhibited with blocking antibodies to the integrin LFA-1, which was shown to be obligate for Th17 cell migration. Therefore, it seems that VLA-4 is required for the entry of Th1 cells to the CNS and that the main target for these cells is the parenchyma of the spinal cord and meningeal layers of the cerebral cortex. On the other hand, Th17 cells require LFA-1 to enter the CNS and, in absence of Th1 cells, primarily traffic to the parenchyma of the

lower brain, cerebellum and brain stem. Ultimately, it appears that the disease phenotype (classical or non-classical) is determined by the ratio of Th17 to Th1 T cells in the CNS (Stromnes, Cerretti et al. 2008, Sie, Korn et al. 2014).

Most of the studies addressing the mechanisms involved in cell migration of Th1 and Th17 populations have been performed using MOG EAE models in C57BL/6 mice. However, a caveat of these models using MOG antigens is the requirement for pertussis toxin (PTX) for the induction of disease. PTX is widely believed to function by opening the blood brain barrier (BBB) to infiltrating CD4 T cells, altering some requirements for adhesion molecule interactions between T cells and endothelial cells that are important for T cell extravasation (Linthicum, Munoz et al. 1982, Yong, Meininger et al. 1993). In addition, evidence exists that PTX can also influence T cell differentiation towards a Th17 phenotype, through direct effects on dendritic cells and other antigen presenting cells (Hofstetter, Grau et al. 2007, Fedele, Spensieri et al. 2010). Interestingly, adoptive transfer models where cells have been Th17 polarized in culture, the addition of PTX at the time of cell transfer has an inhibitory effect on disease (O'Connor, Prendergast et al. 2008, Jager, Dardalhon et al. 2009). Although the reason for this is unclear, it has been hypothesized that Th17 cells might be more susceptible than Th1 cells to PTX mediated chemokine receptor disruption causing them to fail to respond and migrate normally in response chemokine signaling (Su, Silver et al. 2001, Jager, Dardalhon et al. 2009). Therefore, while MOG models are very useful in understanding T and B cell priming and polarization, they may not be the most suitable for studying the mechanisms involved in cell migration.

In contrast, EAE induced with the PLP antigen (PLP<sub>139-151</sub>) does not require the use of PTX. This model is associated with a relapsing-remitting (RR) form of disease,

which is seen in the majority MS patients. Injection of PTX in this model leads to a non-classical disease phenotype, which is similar to that observed with Th17 skewed MOG models described previously. This model was also originally used to characterize the mechanisms driving the effect of anti-VLA-4 treatment in EAE (Theien, Vanderlugt et al. 2001). These studies showed initial efficacy when anti-VLA-4 treatment was given before symptom onset but a worsening of symptoms and relapse rates at later time points. However, no analysis is reported of the “worsening” disease phenotype, in particular to determine whether clinical symptoms were related to classical or non-classical EAE. Furthermore, analysis of the Th phenotype of the disease initiating CD4 cells was not performed, which is likely because the study occurred prior to the discovery of the Th17 phenotype in EAE. In addition, the use of PTX could confound normal trafficking of Th17 cells. In an effort to better understand IVIg effects on the formation and migration of pathogenic Th cells and given what is known about the potentially confounding effects of PTX, we set out to further investigate IVIg’s effects in the RR PPLP<sub>139-151</sub> model of EAE.

### Generation of Hypothesis

A preliminary study was done in the RR PLP<sub>139-151</sub> model to assess IVIg activity in an effort to better understand its mechanism of action with the goal of potentially developing a more effective version of the drug. Unexpectedly we observed non-classical EAE symptoms mice approximately 12-24 hours prior to development of classical EAE in vehicle treated mice. After showing this observation to be reproducible, a dose response study was done to see if the non-classical phenotype was dose dependent and thus



directly related to the amount of IVIg (Fig 1). Indeed the non-classical phenotype was highly correlated with dose of IVIg.

Considering the results of studies described previously showing the influence of IVIg on CAMs and, as described earlier, the influence of adhesion molecules in the location of Th1 and Th17 cells in the CNS, we anticipated that these results were strongly suggestive of an effect of IVIg on the migration of the pathogenic Th1 or Th17 cells to the CNS. In particular, we hypothesized that IVIg prevented Th1 cells from migrating to the CNS, resulting in a preferential location of Th17 cells in the parenchyma of the brain stem and cerebellum. This inhibition may have occurred through the regulation of PSGL-1, LFA-1 and/or VLA-4 expression or activity. This research aims to answer the following three questions in support of our hypothesis:

- 1) Are Th1 and Th17 cell populations modulated in the CNS by IVIg as we hypothesize?
- 2) Are the differences in these populations due to effects of IVIg in the periphery during T cell priming?
- 3) If proven correct about the modulation of Th1 and Th17 cell populations, is this effect due to the specific integrins we hypothesized to be involved, namely VLA-4 and LFA-1.

#### Outline of Studies Conducted

The following studies were designed to bring together several lines of evidence to identify the populations of leukocytes that may have been modulated by IVIg thus leading to the non-classical EAE phenotype we observe. They were designed to examine

both the peripheral lymphoid tissues and the CNS in order to encompass events from early activation and polarization of pathogenic leukocytes to infiltration of the CNS and the results of effector functions of these cells leading to this non-classical phenotype. See Table 1 for the list of studies conducted.

#### Antigen Recall Studies and Phenotype of Peripheral Leukocytes:

(See Table 1: Studies #6 & #8)

As stated previously, activation and polarization of the immune response is the crucial first step in the disease process of EAE. Modulations at this early stage can have profound impact on downstream disease potential and phenotype. IVIg has been shown to interfere with VLA-4 binding to VCAM-1 but additionally it has been shown to have some effects on T cell differentiation in peripheral lymphoid tissue in MOG models of EAE (Theien, Vanderlugt et al. 2001, Othy, Hegde et al. 2013). However there is little evidence for IVIg effects in peripheral lymphoid tissue in this RR EAE model. To first determine whether IVIg affects the induction, proliferative potential and the phenotypes of the Th populations, thus potentially explaining the non-classical disease phenotype we performed an *ex vivo* antigen recall study.

Mice were immunized with PLP<sub>139-151</sub>/CFA and treated every three days with 100 mg/kg of IVIg intraperitoneally (IP). This dose was chosen based on the preliminary study that showed a plateau in the non-classical disease phenotype between 50 and 500 mg/kg (Fig 1). A vehicle-treated control group served as the reference. We looked at antigen-dependent proliferation in the draining (inguinal) lymph nodes (DLNs) associated with the immunization sites and in spleens. Since we wanted to observe the activated and differentiated Th cells before egress from the DLN/spleen and migration to

the CNS, we isolated spleens and DLN cells 9 to 10 days after immunization. Cells from DLNs and spleens from individual mice were restimulated *ex vivo* with increasing concentrations of the PLP<sub>139-151</sub> antigen (0, 1, 10 and 50 µg/ml).

Three sets of measurements were made in these experiments:

- 1) Tritium-labeled thymidine (<sup>3</sup>HTdR) was used to measure leukocyte proliferation. Tritium is a radioactive isotope of hydrogen and the release of beta particles during decay can be used to determine the amount present in a sample. It is incorporated into DNA of growing and dividing cells. By measuring the amount incorporated over a range of antigen concentrations we generated proliferation curves that indicated whether the proliferative potential of the lymphocytes or splenocytes was affected by the IVIg treatment. (*Study #8*)
- 2) We examined a panel of cytokine and chemokines secreted by various Th cells and APCs (including IL-2, IFN $\gamma$  and IL-17) in the supernatants of the proliferating lymphocyte cultures using a multi-plex assay. A relative increase in IL-17 vs IFN $\gamma$  secretion with higher doses of IVIg would indicate increased Th17 differentiation in the DLN or spleen, which might have contributed to explaining our observations in this EAE model. (*Study #8*)
- 3) Finally we wanted to look directly at the phenotypes of the proliferating lymphocytes *ex vivo* by flow cytometry. In particular, we looked at the relative abundance of the IFN $\gamma$  and IL-17-expressing cells to determine if there was a modulation following IVIg treatment. Specifically, an increase in the ratio of Th17 to Th1 cell populations induced by IVIg treatment, might have contributed to explaining the phenotype of disease we observe. (*Study #6*)

Taken together, these measurements should show if IVIg had an effect on the initial steps of T cell priming, activation and differentiation that may contribute to a Th17 skewed phenotype of disease and suggest a possible explanation for the non-classical EAE phenotype. A lack of significant changes in these parameters, however, would be suggestive that IVIg modulates Th activity during the process of trafficking to the CNS.

### Histological Examination of the CNS

*(See Table 1: Study #2)*

In another study we looked directly at leukocyte infiltration in the CNS of classical and non-classical EAE mice. The IVIg mediated disease phenotype that we observed in this EAE model suggested that an increase in inflammation and myelin damage should be observed in lower brain structures and brainstem. Using histology, we set out to confirm the areas of the spinal cord and brain that are affected in each group. Analysis was done at peak of disease when the majority of mice displayed either classical or non-classical EAE.

According to our hypothesis, vehicle-treated mice would show inflammation and demyelination restricted to the spinal cord with brain infiltration limited to the meningeal layers. In contrast, IVIg treatment would be associated with increased infiltration of the cerebellum and brain stem, with preferential parenchymal infiltration and greater damage (demyelination) to the brain and brain stem with IVIg treatment.

Examination of infiltrating leukocytes in the CNS and populations in peripheral lymphoid tissue *(See Table 1: Studies #3, #4, #5, #6 & #7)*

The disease phenotype that we observed in this EAE model suggested a predominant Th17-mediated infiltration, rather than Th1 mediated infiltration of the CNS. In order to confirm this, we proposed identifying the phenotype of the infiltrating Th cells by flow cytometry at onset and peak of disease. Examination of the CNS at peak of disease would show which cell types are most prevalent when the non-classical disease phenotype is maximally expressed while examination of the CNS at onset of disease would provide information on which cell types might be implicated early on before the disease phenotype was observed.

To do this, spinal cords were dissected and infiltrating leukocytes were isolated. Cells were analyzed by flow cytometry for IFN $\gamma$  and IL-17 expression, as well as for infiltrating and resident myeloid cells. If our hypothesis was correct we should have seen an increase in Th17 cells compared to Th1 cells, following IVIg treatment. In parallel we examined the peripheral lymphoid tissues (DLNs and spleens) to see if those Th populations were modulated at those time points. This data would be complementary to the CNS staining, by showing the phenotype of the Th cells before and after trafficking to the CNS

#### mAb blockade of LFA-1

*(Study never conducted because hypothesis was not proven)*

Finally, if we were successful and confirmed that Th17 cells were primarily responsible for the IVIg-mediated phenotype, our goal would then be to show that when Th17 cells are blocked from trafficking by way of their specific integrin LFA-1, ataxic disease is reduced or eliminated. This study design would be similar to that described by

Rothhammer *et al.* (Rothhammer, Heink et al. 2011). The endpoints for this study would be disease phenotype only. No histological or FACS analysis would be performed.

Taken together the results of these studies would be highly informative and add to the knowledge of the mechanism(s) by which IVIg functions. Considering what is published in regards to the influence of IVIg on cell adhesion and migration, as well as its effects on various CAMs as described earlier, it seemed appropriate to further investigate it in a T cell dependent animal model that does not require PTX toxin given its potentially confounding activity on trafficking and extravasation of leukocytes. Additionally, our preliminary data is strongly suggestive of a differential activity of IVIg on Th1 vs Th17 disease phenotype in this model, which could be useful for further investigation of the role these two subpopulations play in autoimmune disease processes in general.

Given that IVIg is a very well tolerated drug with few side effects when compared to anti-integrin therapy, and considering it may have similar activity as anti-VLA-4, if successful, this research could have the potential of being used to identify patients and/or diseases that should be more amenable to IVIg treatment. The potential for patient stratification based on cell surface expression of adhesion molecules may also have direct implications for the diagnosis and treatment of other autoimmune conditions and not only CNS autoimmunity.

## Chapter II

### Materials and Methods

Methods used in this research include EAE induction with PLP<sub>139/151</sub> in female SJL/J mice using a modified protocol developed at Momenta Pharmaceuticals. Leukocyte isolation from CNS by Percoll separation and from peripheral lymphoid tissue by mechanical disaggregation. Flow cytometry was utilized to analyze isolated leukocyte populations. Tritiated thymidine incorporation was utilized to measure cellular proliferation. And multiplex bead assay (Luminex) for measurements of cytokine and chemokine secretion. Statistical analysis of IVIg vs saline treated groups was done using GraphPad Prism v. 6.0 and data was analyzed by unpaired and nonparametric Mann-Whitney Test. Pearson correlations between cytokine secretion and proliferation were done in GraphPad Prism v. 6.0. P values describe the probability of cytokine secretion to be related to cell proliferation.

#### EAE induction, assessment and treatment

EAE was actively induced in 12-13 week old female SJL/J mice (Jackson Laboratories). Mice were kept in accordance with IACUC protocol, which includes supplemental food placed on the floor of the cage and subcutaneously administered lactated Ringer's solution for hydration of incapacitated animals. EAE was induced based on NIH protocol (Miller 2007). Briefly; mice are immunized subcutaneously at two sites on the lower dorsal surface with 100 µg PLP<sub>139-151</sub> emulsified in complete Freund's

adjuvant (CFA), supplemented to a final concentration of 2 mg/ml *M. tuberculosis*. (VWR cat#90002-206 and #90002-208). Mice were monitored daily for signs of disease. Scoring began on day 7. Classical EAE was scored as follows: 1) tail paresis 2) righting reflex delayed or inhibited 3) partial hind limb paralysis 4) complete hind limb paralysis 5) hind limb and fore limb paralysis (requires euthanasia). Non-classical EAE was scored according to the method described by *Guyenet et al* (Guyenet, Furrer et al. 2010). Mice that showed intermediate or a combination of symptoms were noted as Classical/Non-classical. Mice were followed until day 21 for efficacy experiments. Mice requiring euthanasia had the last score prior to euthanasia carried through the study.

For analysis of disease phenotype and histology, seven groups of ten mice were used. Six doses of IVIg were tested (500, 100, 50, 10, 5 and 1 mg/kg) as well as one group as vehicle controls which received saline. IVIg or vehicle were given IP starting on day of immunization then every three days for the duration of the study. For proliferation, polarization, flow cytometry analysis, and CNS leukocyte isolation, two groups consisting of five to twelve mice each were used. Groups included a vehicle control and one IVIg treatment group. IVIg treated mice were dosed IP with 100 mg/kg IVIg in 0.2 ml saline every three days, starting on day 0 and continuing until they were sacrificed for analysis. Vehicle control mice received an equal volume of saline.

#### Proliferation, polarization and cytokine analysis

On day 9 or 10, mice were sacrificed and their draining inguinal lymph nodes (DLNs) and spleens removed aseptically. Single cell preparations were made for each mouse by mechanical disaggregation and passage through a 70-µm nylon filter. Red blood cells from spleens were lysed by ACK lysis buffer (Stem Cell technologies).



Leukocytes for individual mice were counted and resuspended in RPMI 1640 (ATCC), supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were then cultured in 96 well round bottom plates ( $5 \times 10^5$  cells/well in triplicate) for 3 days in the presence of a concentration range of PLP<sub>139-151</sub> (50, 10, 1 and 0 µg/ml). Remaining cells were used for flow cytometry analysis (see below for flow cytometry). After 3 days, supernatants were removed and:

1. Cells were pulsed with 1 µCi of tritiated thymidine (<sup>3</sup>HTdR; PerkinElmer cat# NET027A001MC) for 18 hours. Cells were then harvested and assayed for thymidine incorporation by scintillation counter (PerkinElmer, MicroBeta2®).
2. Supernatants were analyzed by Luminex® 100/200™ (Luminex Corporation). Secretion of thirty two cytokines and chemokines was measured by multiplex (EMD Millipore cat# MCYTOMAG-70K), including IL-2, CCL5, IL-17 and IFN $\gamma$ .

#### Isolation of CNS infiltrating leukocytes

At disease onset (Day 12) and peak of disease (Day 14) mice were anesthetized (ketamine/xylazine) then perfused through the left ventricle with ice cold PBS. Whole spinal cords and brain stems were dissected and mechanically disaggregated then passed through a 70 µm filter to form a cell suspension in RPMI media. Leukocytes were isolated by using a Percoll gradient (30% over 70%). Briefly; in a 15 ml conical tube, cells were suspended in 30% SIP (stock isotonic Percoll) then carefully layered underneath with 70% SIP. Tubes were centrifuged for 20 minutes at 500G at room temperature. Surface debris and lipids were carefully removed and discarded. Then with a transfer pipette, 2-3ml of the 30%/70% interphase (containing the mononuclear cells)

were removed and placed in a clean tube. Cells were then washed 2 times with cold PBS and resuspended in FACS buffer then plated in a V-bottom 96 well plate for staining.

### Flow cytometry

Cells were stimulated in RPMI 1640 culture medium with 50 ng/ml PMA (Sigma-Aldrich), 1  $\mu$ g/ml ionomycin (Sigma-Aldrich) and 1  $\mu$ l/ml monensin (GolgiStop, BD Biosciences) at 37C for 4 hr. Surface staining for T cells and myeloid cells was performed using antibodies to CD3 (clone 14-2C11), CD4 (clone RM4-5), CD8 (clone 53-67), CD11b (clone M1/70), and CD45 (clone 30-F11). After surface staining, cells were fixed and permeabilized (Cytofix/Cytoperm kit, BD), followed by staining with monoclonal antibodies to mouse IL-17 (clone 15-C5) and IFN $\gamma$  (clone G1/57). B cells were not directly stained for in these studies. B cell populations were identified as being CD11b<sup>-</sup>CD45<sup>+</sup>CD3<sup>-</sup> and falling in the lymphocyte gate as determined by FSC/SSC and CD3<sup>+</sup> T cells. All antibodies were obtained from BD Biosciences. Flow analysis was conducted on a FACS Verse (Becton Dickinson) and results were analyzed using the FlowJo software (Tree Star).

### Histology

At peak of disease as determined by score (~ day 14 to 15) mice were sacrificed and cardiac perfused through the left ventricle with ice cold PBS followed by 4% paraformaldehyde. Whole spinal cords (cervical, thoracic and lumbar regions) and brains were analyzed. Brains and spinal cords were prepared separately and fixed in 4% formalin. 7  $\mu$ m sagittal sections were prepared, four sections per mouse (brain and spinal

cord) were analyzed. For light microscopy, sections were stained with hematoxylin and eosin (Sigma-Aldrich). Brain and spinal sections were scored blindly for leukocyte infiltration by a skilled pathologist. Three images per region of the spine (Lumbar, thoracic and cervical) as well as the brain were scored for each section (4 sections per mouse) and averaged. In addition meningeal and parenchymal infiltration were distinguished. An equal number of sections and regions were stained with Luxol fast blue and scored for demyelination.

### Specific Aims

- 1) To demonstrate whether the Th1 and Th17 cell populations we hypothesize to be affected by IVIg are modulated in the CNS.
- 2) To show whether in full or part, the differences in these populations are due to effects of IVIg in the periphery during T cell priming.
- 3) If proven correct about the modulation of these T cell populations, to test whether this effect was due to the specific integrins we hypothesized to be, namely VLA-4 and LFA-1.

Table of Studies Conducted

Study	Study Description	Specific Aims	Techniques	Associated Figures and Tables
# 1	Dose response to IVIg  <b>Rationale:</b> Demonstrate dose dependence of non-classical EAE phenotype	Preliminary data	EAE Induction	<b>Figure 2</b>
# 2	IVIg dose response examining CNS of IVIg treated mice.  <b>Rationale:</b> Demonstrate dose dependence of inflammation and demyelination of anterior CNS with IVIg treatment.	Aim #1	EAE Induction  Histology, H&E staining and Luxol Fast Blue staining	<b>Figure 3</b> <b>Figure 4</b>
# 3	Examine whole spinal cords, DLNs and Spleens at peak of disease (Day 14)  <b>Rationale:</b> To observe cell populations during maximum inflammation in SC. Also analyzed DLN and Spleen populations.	Aim #1 & Aim #2	EAE Induction  Flow cytometry	Same results as Figures 6A and 6B but not shown.
# 4	Examine anterior and posterior sections of spinal cords at peak of disease (Day 14) and DLNs and Spleens.  <b>Rationale:</b> Analyze anterior and posterior regions of SC to get better resolution of the infiltrating leukocytes in SC. Also analyzed DLN and Spleen populations.	Aim #1 & Aim #2	EAE Induction  Flow cytometry	<b>Figure 6C</b>  Same as Figure 5 but not shown  Same results as Figures 6A and 6B but not shown.
# 5	Examine anterior and posterior sections of spinal cords at peak of disease (Day 14).  <b>Rationale:</b> Repeat Study #4 Analyze anterior and posterior regions of SC to get better resolution of the infiltrating leukocytes in SC.	Aim #1	EAE Induction  Flow Cytometry	<b>Figure 5</b>  Same results as Figure 6C but not shown
# 6	Examine whole spinal cords at disease onset (Day 10) and DLNs and Spleens.  <b>Rationale:</b> To observe early infiltrating cell populations in SC, Also examined DLN and Spleen populations.	Aim #1 & Aim #2	EAE Induction'  Flow Cytometry	<b>Figures 6A, 6B and 6D</b>

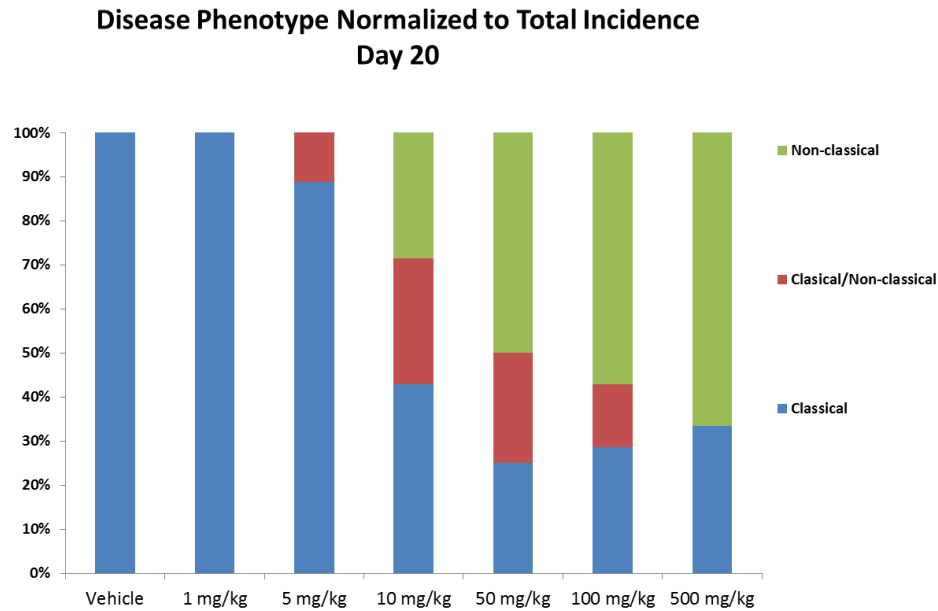
# 7	Examine whole spinal cords at disease onset (Day 12)  <b>Rationale:</b> To observe early infiltrating cell populations in SC	Aim #1	EAE Induction  Flow Cytometry	Described in text only.
# 8	Proliferation Assay on draining inguinal lymph nodes and spleens (Day 9)  <b>Rationale:</b> Characterize proliferation and cytokine/chemokines secretion of lymphocytes to PLP <sub>139-151</sub> . DLN and spleens were isolated and stimulated with antigen, culture media was analyzed for cytokines/chemokines	Aim #2	Proliferation (tritiated thymidine incorporation)  Multiplex Cytokine/Chemokine Analysis.	<b>Figure 7</b> <b>Figure8</b> <b>Table 2</b>

**Table 1: List of studies in this report and rationale**

## Chapter III

### Results

To further understand the mechanism of action of IVIg, we set out to recapitulate several of the observations reported previously for IVIg in other EAE models. Since the use of pertussis toxin (PTX), which alters the permeability of the BBB and influences Th17 differentiation, makes MOG EAE models potentially less relevant to the study of mechanisms involved in cell migration, we decided to address the effects of IVIg in the relapsing-remitting (RR) EAE model using SJL/J mice immunized with PLP<sub>139-151</sub>, which does not require the use of PTX. Mice were dosed prophylactically with various concentrations of IVIg (Study #1), from the day of immunization. As expected, the vehicle group demonstrated classical EAE symptoms. However, a shift in disease phenotype from classical to non-classical (ataxic) disease was observed following IVIg treatment. Higher concentrations of IVIg were associated with increased frequency of non-classical EAE (Fig 2). This phenotype was highly reproducible across three independent studies and represents a novel observation for IVIg treatment.

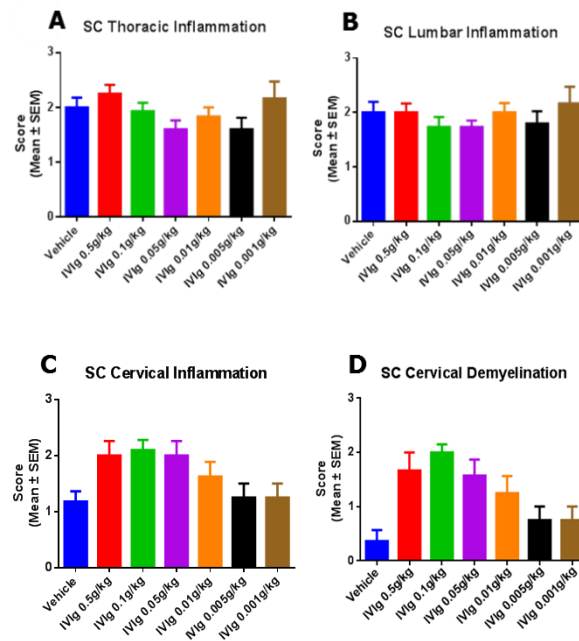


**Figure 2. Disease phenotype with IVIg treatment.** Frequency of mice displaying non-classical EAE (green), mixed classical and non-classical (red) or classical (blue) disease phenotype.

Since non-classical (ataxic) EAE is associated with increased inflammation of the anterior spinal cord and lower brain structures we decided to further investigate this shift in disease phenotype by examining the CNS of mice with classical and non-classical EAE by histology. Mice were induced with PLP<sub>139-151</sub> and treated with various concentrations of IVIg as in the previous study (Study #2). Five animals from each group were sacrificed at peak of disease (Day 14), and prepared for histological examination of the cervical, thoracic and lumbar regions of the spine as well as the brain. Histological sections were stained and scored for infiltrating leukocytes and demyelination.

No significant changes were observed compared to vehicle controls with IVIg treatment in either the thoracic or lumbar sections as measured by leukocyte infiltration (Fig 3A & 3B) or demyelination (not shown). However, there was an IVIg dose-

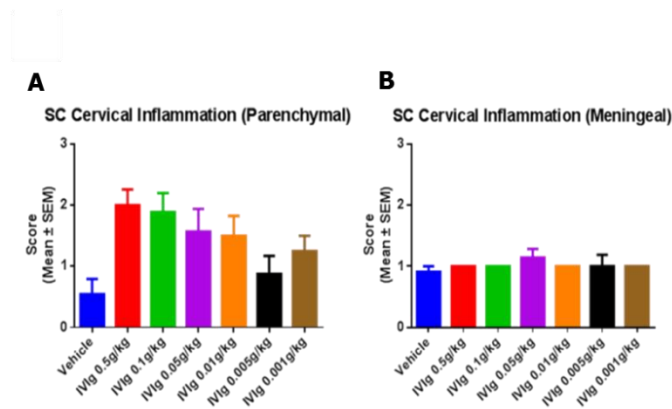
dependent increase in infiltrating leukocytes as well as an increase in demyelination in the cervical region of the spinal cords (Fig 3C & 3D).



**Figure 3. Inflammation and demyelination of spinal cord (SC) regions.** Inflammation scores for A) thoracic or B) lumbar regions and C) cervical regions of the SC. D) Shows demyelination scores for the cervical SC region.

Furthermore, there was an IVIg dose dependent increase in the parenchymal infiltration of leukocytes compared to meningeal infiltration (Fig 4). This is consistent with what has been described previously for non-classical EAE, showing increased lymphocyte infiltration of the parenchyma of the anterior spinal cord and lower brain structures. This is also consistent with our hypothesis of increased infiltration of the anterior spinal cord and lower brain structures.





**Figure 4. Parenchymal and meningeal inflammation of the cervical spinal cord (SC).** Inflammation of the A) parenchyma and B) meninges of the cervical SC region.

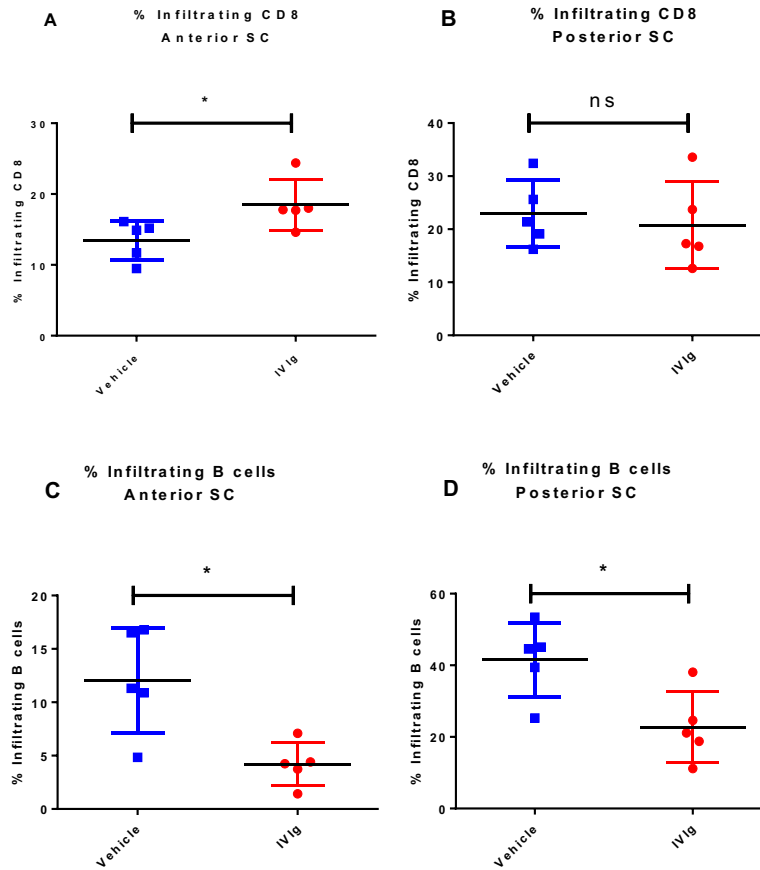
Next, in order to identify the cell populations involved with the inflammation we observed by histology, we isolated the inflammatory immune cells from spinal cords of EAE mice at peak of disease (day 14) and analyzed them by flow cytometry (Study #3). Based on the previous dose response studies we chose the 100 mg/kg dose (Fig 2 & 3). Cell populations examined by flow cytometry included CD4<sup>+</sup> and CD8<sup>+</sup> T cells and microglia. B cells were estimated to be the CD45<sup>+</sup>CD3<sup>-</sup>CD11b<sup>-</sup> population. CD4<sup>+</sup> T cells were further examined for IFN $\gamma$  (Th1) and IL-17 (Th17) expression. Microglia could be further divided into parenchymal or perivascular based on the level of CD45 expression (Rawat and Spector 2016).

No significant modulation of Th1 or Th17 cell populations was observed with IVIg treatment (See Appendix 1). In addition there were no changes in the CD8 or microglial populations. One possibility for this result is that by isolating leukocytes from the entire spinal cord, differences in populations that are regional might not be observable. Given the large number of infiltrating leukocytes observed in the whole

spinal cord at peak of disease, small differences in the anterior or posterior portions of the spinal cord may be difficult to measure.

The non-classical phenotype we observed should be associated with increased inflammation of the anterior portions of the spinal cord and since our histological evidence confirms this in our model, we next analyzed the spinal cord in two sections with the thought that better resolution of the infiltrating populations might be observed. To do this we divided the spinal cords into anterior and posterior sections then isolated leukocytes separately (Studies #4 and #5).

We did not observe a shift in the numbers of IFN $\gamma$  expressing Th1 cells or IL-17 expressing Th17 cells in either the anterior or posterior regions of the spinal cords. Neither was the ratio of Th1/Th17 affected as reported by Stromnes et al. who showed that an increase in the ratio of Th17 to Th1 cells was responsible for the non-classical phenotype they observed (Stromnes, Cerretti et al. 2008). However we did observe changes with IVIg treatment, including an increased frequency of infiltrating CD8<sup>+</sup> cells in the anterior portion of the SC (Fig 5A). In addition the frequency of infiltrating B cells was decreased in both the anterior and posterior SC regions (Fig 5C & D). CD8<sup>+</sup> populations remained unchanged in the posterior region of the SC (Fig 5B). These results were observed in two independent experiments.

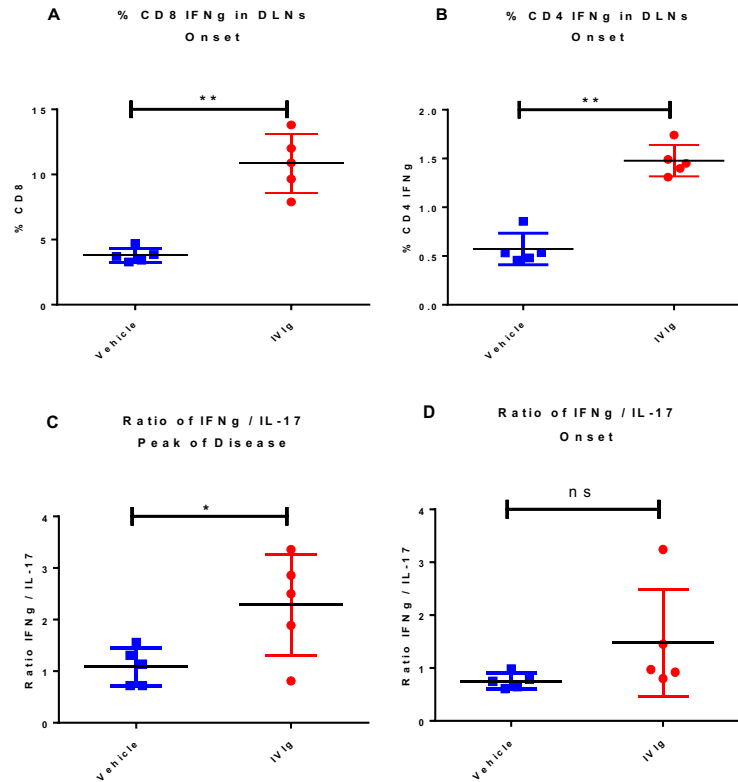


**Figure 5. Cell Populations modulated in the anterior and posterior SC:** Frequency of: A) CD8 cells anterior SC; B) CD8 cells posterior SC; C) B cells anterior SC; and D) B cells posterior SC. Statistical significance was addressed using a Mann-Whitney Test (\* > 0.05).

Since differences in early infiltrating cell populations might be responsible for the non-classical phenotype and that by peak of disease these differences might not be observable due to the large influx of leukocytes at that time point, we next examined the SCs of EAE mice at onset of disease (Day 12, Study #7). A difference in Th1 and Th17 infiltration at onset might set up the conditions for non-classical EAE to occur which would support our hypothesis. However similar to our observation at peak of disease, we did not see a modulation of Th1 or Th17 populations when examining SCs at onset of disease. No differences were observed in infiltrating CD8, microglia or B cells as well.

Although we did not observe differences in the Th1/Th17 composition of the spinal cord, differences in these populations in the periphery might be indicative of how IVIg is functioning to cause the non-classical EAE phenotype. To further understand the effects IVIg may be having on leukocytes in the periphery in EAE, we investigated the cellular composition of the spleen and draining lymph nodes (DLNs) by flow cytometry (Studies #3, #4 and #6). In particular by measuring these populations in the DLNs we would see if IVIg was having an effect on the activation and differentiation of these populations. As in the spinal cords we looked specifically at the IFN $\gamma$  and IL-17 expressing populations as well as the CD8<sup>+</sup> and myeloid populations.

We were unable to observe any differences between IVIg and vehicle when examining the spleens. However the IVIg treatment was associated with an elevated frequency of IFN $\gamma$ -expressing CD8<sup>+</sup> (Fig 6A) and CD4<sup>+</sup> (Fig 6B) cells in the DLNs at both onset and peak of disease. In addition, we observed a significant increase (approximately 2:1) in the ratio of Th1 (IFN $\gamma$ ) vs Th17 (IL-17) cells at peak of disease, which was not significant at disease onset (Fig 6C & 6D). This contrasts with the observations of Othy et al who showed a decrease in both Th1 frequency in the DLNs of IVIg-treated mice (Othy, Hegde et al. 2013).

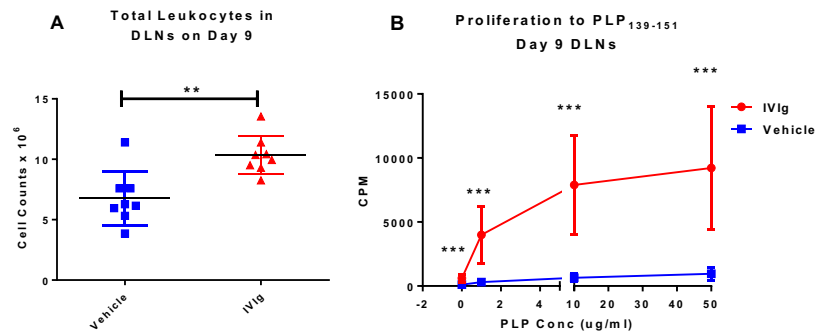


**Figure 6. Frequency of cell populations modulated in DLNs at onset and peak of disease:** A) IFN $\gamma$ -expressing CD8 cells; B) IFN $\gamma$ -expressing CD4 cells; Ratio of IFN $\gamma$  / IL-17-expressing CD4 cells at C) peak of disease and D) disease onset. Statistical significance was addressed using a Mann-Whitney Test (\* < 0.05, \*\* < 0.01).

One of the key steps in actively induced autoimmune models such as EAE is the priming and polarization of antigen specific lymphocytes in the periphery. Modulations at this step can have profound effects on the type of disease that is induced and the cell populations involved. To investigate the potential that the activity of IVIg in our model is at least partially mediated by the priming and activation of lymphocytes in the periphery, we conducted a proliferation assay to address early events of antigen presentation and lymphocyte activation (Study #8).

Our results showed significantly greater numbers of leukocytes were isolated from the DLNs of the IVIg treated group. In addition, while no significant differences in proliferation in the spleens of vehicle and IVIg-treated mice were observed, there was a

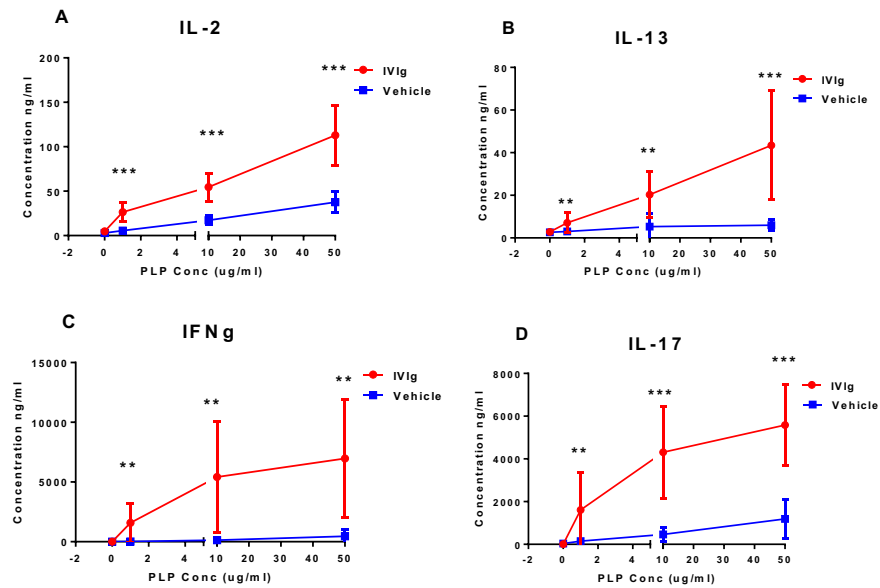
highly significant increase in proliferation of the DLNs of IVIg-treated mice. (Fig 7A & 7B). This is a novel observation and the first time to our knowledge that IVIg has been shown to increase antigen specific proliferation in an animal model of inflammation.



**Figure 7. Proliferation of DLN cells to PLP<sub>139-151</sub>:** A) Proliferation IVIg and vehicle DLN cells to the PLP<sub>139-151</sub> antigen. B) Number of cells isolated from DLNs of vehicle and IVIg treated mice. Statistical significance was addressed using a Mann-Whitney Test (\*\* < 0.01, \*\*\* < 0.001).

Culture supernatants were also analyzed for cytokine & chemokine production (Study #8). Th1 (Fig 7C), Th2 (Fig 7B) and Th17 (Fig 7D) associated cytokines and chemokines were significantly upregulated in the IVIg-treated group, compared to vehicle controls. To test for the degree of correlation between analyte secretion and proliferation, correlations between analyte secretion and proliferation (cpm) were done and a Pearson correlation coefficient generated (Table 2). Most analytes correlated well with proliferation for both IVIg and vehicle treated groups indicating that even though there was less secretion of these cytokines/chemokines in the vehicle group, what was observed was antigen specific. Differences between the groups was a matter of degree and likely could be explained by the increase in proliferation. However, RANTES (aka CCL5) levels only correlated with antigen specific proliferation in IVIg-treated DLNs ( $p < 0.0001$ ) but not with vehicle ( $p = 0.259$ ). CCL5 is a Th1 chemokine also secreted by

APCs. It is a powerful chemo-attractant for many types of leukocytes towards sites of inflammation including T cells, B cells, monocytes, macrophages and NK cells (Miyagishi, Kikuchi et al. 1997). Correlation of this chemokine with proliferation indicates there is a Th1 population of cells being generated in the DLNs with IVIg treatment that is not present in the vehicle group. The secretion of CCL5 in the vehicle group is therefore most likely due to APC secretion alone. This is consistent with the observed increase in IFN $\gamma$  expressing CD4<sup>+</sup> cells in the DLNs.



**Figure 8. Selected cytokines secreted in PLP<sub>139-151</sub> concentration dependent manner:** A) IL-2, B) IL-10 Th2 cytokine, C) IFN $\gamma$  Th1 cytokine, D) IL-17 Th17. Statistical significance was addressed using a Mann-Whitney Test (\* < 0.05, \*\* < 0.01, \*\*\* < 0.001).

Correlation to Counts					
Cytokine	Source in LN	r IVIg	p-value	r Vehicle	p-value
IFN $\gamma$	Th1	0.763	<0.0001	0.504	0.003
IL-17	Th17	0.858	<0.0001	0.611	0.002
IL-2	Th1	0.798	<0.0001	0.854	<0.0001
GM-CSF	Th17	0.767	<0.0001	0.591	0.0004
<b>RANTES (CCL5)</b>	<b>Th1, APC</b>	<b>0.676</b>	<b>&lt;0.0001</b>	<b>0.259</b>	<b>ns</b>
IL-3	Th1, Th2, Th17	0.645	<0.0001	0.625	0.0001
IL-6	Th1	0.866	<0.0001	0.714	<0.0001
IL-13	Th2	0.763	<0.0001	0.5037	0.0033
KC	APC, endothelium	0.770	<0.0001	0.394	0.03
MIP-1a	Th1, APC	0.535	0.0016	0.6517	<0.0001
MIP-1b	APC, CD8	0.584	0.0005	0.7899	<0.0001

**Table 2. Cytokines and chemokines upregulated in a PLP<sub>139-151</sub> concentration dependent manner in DLNs:** r = Pearson correlation values of analyte concentration to proliferation (cpm) for IVIg and vehicle. P-value is the significance of Pearson correlation of analyte to proliferation (cpm).

### Summary of Results

IVIg treatment in PLP<sub>139-151</sub> actively induced EAE leads to a dose-dependent increase of a non-classical disease phenotype. This is associated with increased leukocyte infiltration of the cervical spinal cord and brain. Specifically leukocytes are found primarily in the parenchyma of the neural tissue. Upon further investigation of the SC by flow cytometry, and contrary to our initial hypothesis, we did not observe a modulation of Th1/Th17 populations by IVIg in the SC either at disease onset or peak of disease. However we did observe a significant decrease in B cells throughout the whole SC with IVIg treatment and an increase in the frequency of CD8<sup>+</sup> cells in the anterior region.

When we examined peripheral lymphoid tissue we observed a general increase in the total number of leukocytes in the DLNs of IVIg treated mice compared to vehicle controls. Flow cytometry analysis of peripheral lymphoid tissue showed an increase in CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup> as well as CD8<sup>+</sup> IFN $\gamma$ <sup>+</sup> expressing cells in DLNs at onset and peak of disease. We also observed an increase in the ratio of IFN $\gamma$ /IL-17 cells at peak of disease only.



Upon recall with PLP<sub>139-151</sub>, IVIg treated lymphocytes showed a highly significant increase in proliferation compared to vehicle controls and secreted significantly more Th1, Th2 and Th17 cytokines and chemokines in a PLP<sub>139-151</sub> antigen-specific manner. This secretion correlated well with proliferation in both IVIg and vehicle treated mice (proliferation vs. concentration). In contrast the Th1 chemokine RANTES showed no correlation with proliferation in vehicle treated mice but a highly significant correlation with IVIg treatment.

## Chapter IV

### Discussion

Our hypothesis was based on a novel observation that treatment of PLP<sub>139-151</sub> induced RR EAE with IVIg led to a dose dependent increase in non-classical (ataxic) disease phenotype. To our knowledge this has not been previously described for IVIg in any model of EAE. Based on observations by several independent laboratories that demonstrated a shift from a predominantly Th1 response to a predominantly Th17 response led to a similar shift in disease phenotype we further developed our hypothesis. Specifically an increase in the ratio of Th17 to Th1 cells infiltrating the CNS was shown to be crucial for this observation (Stromnes, Cerretti et al. 2008, Jager, Dardalhon et al. 2009, Glatigny, Duhen et al. 2011, Rothhammer, Heink et al. 2011). Our theory was bolstered by observations that IVIg treatment led to a decrease in VLA-4 dependent rolling and adhesion *in vitro* and *in vivo* (Lapointe, Herx et al. 2004, Gill, Doig et al. 2005). Since integrins such as VLA-4 and LFA-1 are differentially expressed on Th1 and Th17 cells respectively, and since VLA-4 was shown to be necessary for Th1 cells to migrate to the CNS, we hypothesized that this might explain our observations (Stromnes, Cerretti et al. 2008, Rothhammer, Heink et al. 2011).

The results of the studies we conducted failed to demonstrate a shift in the Th17 versus Th1 populations in the CNS of non-classical EAE mice, therefore our original

hypothesis was not confirmed. Notwithstanding this, we did make some significant observations.

### Major Observations

There were three major observations to come from these studies:

- 1) We observed a depression in the B cell population found in the spinal cords of IVIg treated mice.
- 2) We observed an increase in the CD8<sup>+</sup> population of lymphocytes in the anterior SC of IVIg treated mice that is associated with increased inflammation and demyelination of the neural tissue in that region.
- 3) We observed increased antigen specific proliferation and cytokine/chemokine expression in the DLNs associated with IVIg treatment, as well as an increase in the frequency of Th1 cells and increased expression of the Th1 chemokine CCL5.

### Suppression of B cells in the CNS with IVIg Treatment

Although there has been a lot of attention in MS research paid to the contribution of T cells and particularly CD4 cells, it is well known that many other immune cells play

critical roles in MS. B cells play a role in both worsening and ameliorating symptoms of MS and EAE. While it was originally believed that their main function in autoimmune disease was to produce auto-antibodies that targeted self-tissues, B cells are now known to play multiple roles ranging from pro-inflammatory to the more recently described regulatory B cells (Bregs) that can either exacerbate or ameliorate autoimmune diseases, including MS and EAE (Wolf, Dittel et al. 1996, Matsushita, Yanaba et al. 2008, Weber, Prod'homme et al. 2010, Mann, Ray et al. 2012).

Pro-inflammatory B cells can mediate their function through autoantibodies, cytokine secretion and antigen presentation. Autoantibodies target self-tissues for further Fc mediated cytotoxicity and demyelination of axons by macrophages and microglia. In addition, B cells also secrete pro-inflammatory cytokines and chemokines that can exacerbate T cell effector function leading to increased pathogenicity at sites of inflammation. They can also act as APCs in both the CNS and peripheral tissues to both initiate and reactivate T cells via major histocompatibility complex class II (MHC II) (Weber, Prod'homme et al. 2010, Molnarfi, Schulze-Topphoff et al. 2013). Appreciation for the pathogenicity of B cells has increased with the identification of ectopic germinal center-like structures in the CNS of MS patients, and their association with the progression of disease from relapsing remitting to secondary progressive MS (SP MS). (Serafini, Rosicarelli et al. 2004, Rovituso, Scheffler et al. 2016).

Regulatory B cells are also known as Bregs or B10 cells due to their expression of the anti-inflammatory cytokine IL-10. Although they show some heterogeneity in the markers they express, in general they are described as CD19<sup>+</sup>CD5<sup>+</sup>CD1d<sup>hi</sup> in mice and express the anti-inflammatory cytokines TGF- $\beta$  and IL-35 in addition to IL-10 (Fillatreau, Sweenie et al. 2002, Matsushita, Yanaba et al. 2008). Bregs have been shown

to suppress the functions of Th1 and Th17 cells while promoting the expansion of Tregs. The loss of Breg populations or functions are directly associated with worse disease outcome in EAE and relapses in MS patients (Wolf, Dittel et al. 1996, Matsushita, Yanaba et al. 2008, Knippenberg, Peelen et al. 2011, Han, Sun et al. 2016). It has now been shown that Bregs arise early in active induction models of EAE (Matsushita, Horikawa et al. 2010).

In light of this, our observation that there is a significant decrease in B cell infiltration of the spinal cord in IVIg treated mice is both novel and especially interesting. A deficit in the Breg population with IVIg treatment could explain the more severe disease that these mice develop. In both MOG<sub>35-55</sub> and PLP<sub>139-151</sub> it has been shown that antigen specific Breg cells arise that express IL-10 (Matsushita, Yanaba et al. 2008, Oxombre, Lee-Chang et al. 2015). They are found to rapidly expand in the spleen and migrate to the CNS where they influence the severity of symptoms early in disease progression. They have been shown to suppress Th1 and Th17 cell differentiation (in particular IFN $\gamma$  expression) and enhance Th2 polarization (Lampropoulou, Hoehlig et al. 2008, Matsushita, Horikawa et al. 2010). What is noteworthy is that neither PLP<sub>139-151</sub> nor MOG<sub>35-55</sub> models of EAE generate pathogenic B cells that play a part in disease progression since they are not efficient B cell antigens (Matsushita, Yanaba et al. 2008, Marin, Eixarch et al. 2014). This could indirectly suggest the missing B cell population in our model is regulatory in nature.

If this turns out to be the case, the question becomes how does IVIg cause this deficit in the infiltrating Breg population? There are at least two potential mechanisms, which are not mutually exclusive, by which this may occur. IVIg may have a direct effect on the

formation or function of this Breg population and/or, as we hypothesized with Th1 cells earlier, IVIg may interfere with Breg migration.

To the first possibility, IVIg has been shown to have direct inhibitory activity on B cells *in vitro* and *ex vivo*. In two studies, one in murine B cells, another from B cells isolated from patients with lupus erythematosus (SLE), it was shown that IVIg inhibits IL-10 and IL-6 secretion. This led to BCR desensitization, and altered downstream signaling and to an overall B cell functional silencing or an anergic like state that was reversible. If this could be shown to occur in the PLP<sub>139-151</sub> model it may indicate that Breg differentiation or function has been inhibited by IVIg leading to an overall depression in the Breg population that leads to the lower frequency observed in the CNS (Kessel, Peri et al. 2011, Seite, Goutsmedt et al. 2014).

To the second possibility, it remains possible IVIg mediates its effects through VLA-4. As described previously, IVIg has been shown to modulate cellular adhesion molecule interactions and specifically VLA-4 (Lapointe, Herx et al. 2004, Gill, Doig et al. 2005, Anthony, Wermeling et al. 2008). Although we did not observe a modulation of CD4 cells in our model as we predicted, it still remains a possibility that IVIg is affecting B cells via VLA-4. In two recent studies VLA-4 has been shown to be necessary for Bregs to migrate to the CNS in MOG<sub>35-55</sub> EAE (Glatigny, Wagner et al. 2016, Lehmann-Horn, Sagan et al. 2016). Conditional knockout of VLA-4 in B cells in both studies led to decreased accumulation of CD1d<sup>hi</sup> expressing B cells in the CNS that was associated with more severe disease. There was no difference in the frequency of Th1 or Th17 cells in the CNS however. Additionally, Glatigny et al. showed VLA-4 knockout led to increased CD4<sup>+</sup> cells in the DLNs (Th1 and Th17) as well as increased proliferation to MOG<sub>35-55</sub>

suggesting VLA-4 mediated activity in the periphery. These results closely mirror our observations in PLP<sub>139-151</sub> induced EAE with IVIg.

If confirmed that the CD11b<sup>+</sup>CD45<sup>+</sup>CD3<sup>-</sup> population we observe depressed in the spinal cords of IVIg treated mice are indeed Bregs, it may explain in part our observations of clinically more severe disease in these mice. It may also help explain another major observation we made of an increase of CD8<sup>+</sup> cells in the anterior portion of the spinal cords of these mice associated with greater demyelination.

#### IVIg Leads to Increased Frequency of CD8<sup>+</sup> Cells in the Anterior Spinal Cord

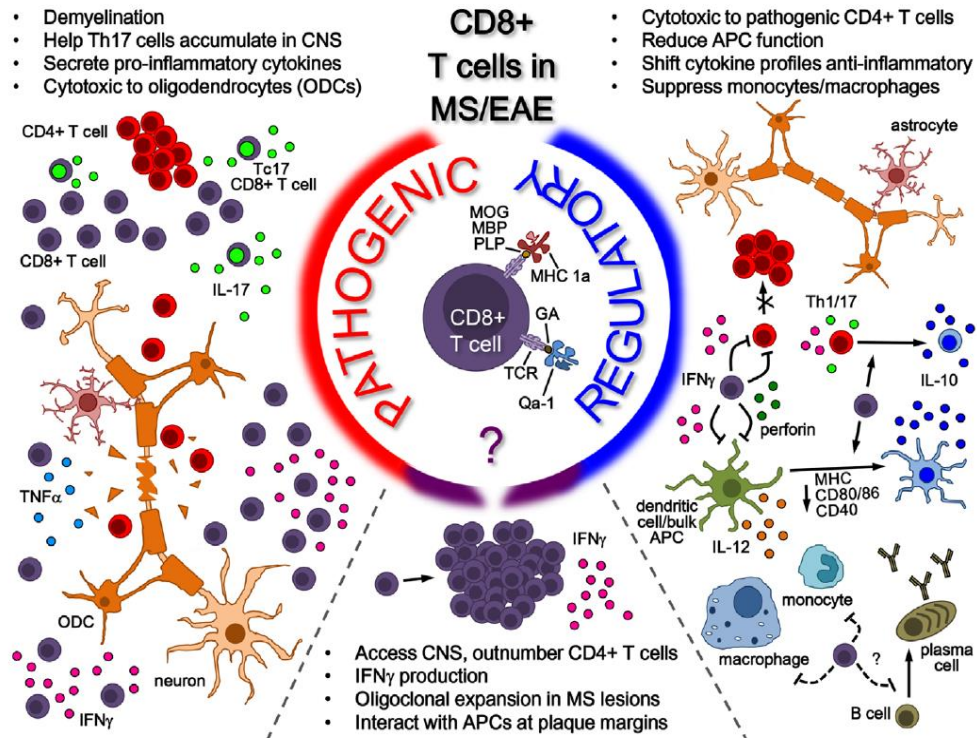
As with B cells and CD4<sup>+</sup> T cells, it is now recognized that CD8<sup>+</sup> cells consist of both pro- and anti-inflammatory populations. Classically, CD8<sup>+</sup> T cells, also known as cytotoxic lymphocytes (CTLs), have been considered pro-inflammatory and the population of lymphocytes that directly or indirectly kill other cells expressing aberrant antigenic peptides in context of MHC Class I (MHC I).

CTLs are highly implicated in demyelination and axonal damage in MS patients. They can be found in greater numbers than CD4<sup>+</sup> cells in perivascular cuffs near sites of MS lesions. Histologically they have been found with their cytolytic granules polarized towards oligodendrocytes and demyelinated axons indicative of imminent T cell mediated killing in both MS and EAE. Additionally, when examined in the CNS of MS patients and in EAE, they are oligoclonal indicating they are proliferating to a common antigen lending support to the requirement of neuroantigen reactivation at the site of

inflammation (Hauser, Bhan et al. 1986, Babbe, Roers et al. 2000, Bitsch, Kuhlmann et al. 2000). In general they express IFN $\gamma$  but it has recently been shown that a subset, expressing IL-17, is associated with active CNS lesions in MS patients more so than their IFN $\gamma$  expressing counterparts (Tzartos, Friese et al. 2008, Saxena, Martin-Blondel et al. 2011).

Complementary to CTLs are the more recently described regulatory CD8<sup>+</sup> cells or CD8<sup>+</sup> Tregs. These cells have been demonstrated to perform many anti-inflammatory functions including inhibiting or killing pro-inflammatory CD4<sup>+</sup> cells, downregulating or anergizing APCs, promoting anti-inflammatory cytokine production and suppression of pro-inflammatory monocyte and macrophages (Ortega, Kashi et al. 2013, Sinha, Boyden et al. 2015). CD8<sup>+</sup> Tregs are phenotypically quite diverse and less clearly defined and understood than CTLs or B cells. Many express IL-10 but not all and some share many of the same features as CTLs. This heterogeneity in CD8 Treg phenotype makes them difficult to distinguish from CTLs unless their effector function is examined (See Fig 9) (Oliveira, van Veelen et al. 2010, Sinha, Boyden et al. 2015).





**Figure 9. Pathogenic and Regulatory roles of CTLs and CD8<sup>+</sup> Tregs.** (Adapted from Sinha et al.) (Sinha, Boyden et al. 2015)

As mentioned with B cells earlier a distinction between CTLs and CD8 Tregs is important in our case. Our results show a significant increase in frequency of infiltrating CD8<sup>+</sup> cells localized to the anterior portion of the spinal cords of IVIg treated mice (Fig 4A). Their prevalence was associated with increased inflammation and demyelination observed in that region of the CNS (Fig 2D). Interestingly it has also been shown that CTL mediated EAE leads to severe demyelination in MOG<sub>35-55</sub> EAE and non-classical disease presentation with leukocyte infiltration mainly restricted to the brain and brainstem (Huseby, Liggitt et al. 2001, Sun, Whitaker et al. 2001). These are similar to features we observe with IVIg treated PLP<sub>139-151</sub> EAE both in terms of disease phenotype and histology and might be indicative of CTL mediated killing of oligodendrocytes in our model.

It has been widely shown and well accepted that CTLs require restimulation by antigen at the site of effector function in order to mediate cell killing and do not engage in non-specific bystander activity (McGavern and Truong 2004). Therefore our hypothesis that the increase in frequency of CD8<sup>+</sup> cells in the anterior region of the CNS are due to increased CTLs, and are responsible for the demyelination we observe, requires them to be restimulated via MHC I. Previously PLP<sub>139-151</sub> has been reported to be MHC II restricted and thus unable to be presented to CD8<sup>+</sup> cells via MHC I. However recently there has been a report of CTLs being generated in wild type SJL mice to the PLP<sub>139-151</sub> antigen although in small numbers (Ortega, Kashi et al. 2015). If this proves true and the infiltrating CD8<sup>+</sup> cells we observe are shown to be CTLs and not CD8 Tregs then it may help explain what we observe in the anterior region of the CNS in terms of inflammation as well as the non-classical disease phenotype (Ortega, Kashi et al. 2015).

Our observations on B cells and CD8<sup>+</sup> cells may be interrelated. Although few people have studied the effects of Bregs on CTLs in EAE and MS, it has been demonstrated that Bregs exert some influence over cytotoxic CD8<sup>+</sup> cells via IL-10 production (Siewe, Stapleton et al. 2013). In addition it has been shown that TGF- $\beta$  expressing B cells directly induced anergy in CD8<sup>+</sup> cells (Parekh, Prasad et al. 2003). This could have direct implications for our observations, namely that a reduction of Bregs in the CNS is facilitating increased CTL oligoclonal proliferation and effector function in the anterior CNS leading to greater demyelination and non-classical presentation.

#### IVIg Effects on Proliferation and Cytokines/Chemokines in DLNs

In conjunction with our B cell and CD8<sup>+</sup> cell observations, we consistently observed both a significant increase in the size of the draining inguinal lymph nodes and the total number of leukocytes isolated following IVIg treatment. In addition we observed that these lymphocytes proliferated to a greater extent to PLP<sub>139-151</sub> and concurrently showed an increase in secretion of a wide range of cytokines and chemokines. These observations could be explained in multiple ways that are not mutually exclusive.

One possibility is that we are making a similar observation to Othy et al. who showed that IVIg treatment in the MOG<sub>35-55</sub> model led to significantly larger DLNs as measured by total cells isolated as well as a significant increase in the CD4 population (Othy, Hegde et al. 2013). Upon further investigation it was determined that sphingosine-1-phosphate receptor (S1P1), which is required for CD4<sup>+</sup> cell egress from the DLN and downstream signaling through mTOR pathway, were down-regulated on the CD4<sup>+</sup> population. This inhibition was shown to be mediated by the Fab' portion of IVIg but how exactly IVIg induces this down-regulation is still unknown. Such inhibition following IVIg treatment could explain why upon restimulation with PLP<sub>139-151</sub>, we observed significantly greater proliferation compared to vehicle control mice. The interpretation being that the lymphocytes from the DLNs of the IVIg treated mice contained a higher percentage of CD4<sup>+</sup> T cells (PLP<sub>139-151</sub> specific as well as non-specific CD4<sup>+</sup> cells). This would lead to more antigen specific CD4<sup>+</sup> cells of all Th subsets in the DLNs that could proliferate and secrete Th1, Th2 and Th17 cytokines. However, in contrast to our results Othy and others observed a decrease in secretion of IFN $\gamma$ , IL-17 and IL-2 (Othy, Hegde et al. 2013, Figueiredo, Drohomysrecky et al. 2014). So although there might be an S1P1 mediated increase in DLN cellularity due to decreased egress of CD4<sup>+</sup> cells, in our case there seems to be something else going on as well that may not be mutually exclusive given IVIg's pleotropic nature.

Once again VLA-4 is potentially implicated in our observations. In light of our original hypothesis and the extensive evidence showing IVIg's interactions with adhesion molecules and in particular VLA-4, the results of Theien et al. are particularly interesting. Using the anti-VLA-4 mAb PS/2 for treatment of the PLP<sub>139-151</sub>/SJL EAE model, they showed a significant increase in lymphocyte proliferation to the PLP<sub>139-151</sub> antigen compared to control ab treated mice. In addition there was a significant antigen mediated increase in IFN $\gamma$  secretion by these cells. This closely resembles our result in the same strain of mouse with the same antigen. As with our result it would still be possible that this was due in part to inhibition of CD4<sup>+</sup> egress from the DLN (Theien, Vanderlugt et al. 2001). This is also consistent with what Glatigny et al. observed for MOG<sub>35-55</sub> in VLA-4 knockout mice as mentioned earlier (Glatigny, Wagner et al. 2016).

In addition to the important role integrins such as VLA-4 and LFA-1 play in migration, they also are necessary for formation of the immune synapse during antigen presentation to T cells. During normal antigen presentation of B cells or DCs to T cells, VLA-4 and LFA-1 colocalize to the peripheral supramolecular activation complex (pSMAC) in both mice and humans. Mittelbrunn et al. showed when a blocking antibody to VLA-4 is used, it causes VLA-4 to localize with CD3 at the center of the immune synapse. This results in Th1 polarization of the T cell with increased IFN $\gamma$  expression. Mittelbrunn went on to show in a Th2 driven disease model in rats (autoimmune nephritis) that anti-VLA-4 treatment skews the immune response to Th1 phenotype with increased levels of IFN $\gamma$  found in serum. Although no similar study has been reported examining IVIg's effects on integrin localization in the immune synapse, if similar activity could be demonstrated it would support our results and would be a novel observation for IVIg and potentially why the Th1 chemokine CCL5 is upregulated relative to vehicle controls in our model (Mittelbrunn, Molina et al. 2004).

As mentioned previously, CCL5 is an important chemokine that is expressed by Th1 cells and various APCs. It is one of several chemokines described as necessary for T cell migration to the CNS. Its increased secretion in a PLP<sub>139-151</sub> concentration dependent manner indicates that IVIg is inducing a CCL5 expressing Th population that is not seen in the vehicle lymphocytes whether or not egress is inhibited from the DLNs. Since the secretion of CCL5 in vehicle mice is low and does not increase with PLP concentration, it is likely background secretion from APCs in the culture.

This is significant because CCL5 secreting T cells are amongst the first cells seen to cross the BBB in actively induced EAE and are found localized to the subarachnoid space and pia matter of the choroid plexus (Miyagishi, Kikuchi et al. 1997, Wilson, Weninger et al. 2010). It has also been shown that ablation of CCR1, one of the receptors for CCL5, completely inhibits EAE and that CCL5 is required for influx of leukocytes in EAE (Rottman, Slavin et al. 2000, dos Santos, Barsante et al. 2005). Finally CCL5 has been shown to be expressed in active lesions of MS patients and is considered a biomarker of active disease in cerebral spinal fluid (CSF) (Sorensen, Tani et al. 1999, Tomioka and Matsui 2014) The implication of our result being that IVIg, by inducing an increase in CCL5 expressing Th1 cells, might trigger a preferential localization of leukocytes in the anterior SC and lower brain structures, which may help to explain the aggressive leukocyte influx (including CD8<sup>+</sup> T cells) and demyelination we observe in those areas leading to the non-classical disease phenotype we observe.

IVIg Effects on Frequency of Th1, Th17 and CD8 Populations in the DLNs

Although we did not see a shift in the Th1 and Th17 populations in the spinal cords we did see a shift in the DLNs with an increase in the frequency of Th1 cells relative to Th17 cells as determined by their expression of IFN $\gamma$  and IL-17. This is the opposite of the ratio observed in the spinal cords by Stromnes et al. that was associated with non-classical EAE. If indeed activation and polarization are being affected by IVIg in the DLNs, then IVIg may be having an effect on the APCs responsible for this. In DLNs, the majority of those APCs are B cells. As pointed out earlier, IVIg has been shown to have direct effects on B cells, leading to an inhibition of their pathogenic potential and possibly anergy. Similar effects have been shown for other APCs too. This may lead to increased Th1 polarization. In particular if IVIg inhibits integrin binding during APC antigen presentation and activation, similar to what is observed with anti-VLA-4, this might lead to an increase in Th1 cells as shown by Glatigny and Mittelbrunn and explain the increase in IFN $\gamma$  expressing CD4<sup>+</sup> and CD8<sup>+</sup> cells we observe (Mittelbrunn, Molina et al. 2004, Glatigny, Wagner et al. 2016).

### Summary

In summary there were three major observations to come out of our research. 1) A depression in the B cell population found in the spinal cords of IVIg treated mice and the hypothesis that these cells may be Bregs in nature. 2) An increase in the CD8<sup>+</sup> population of lymphocytes in the anterior SC of IVIg treated mice that is associated with increased inflammation and demyelination of the associated CNS tissue implying they may be CTLs. And 3) the observation of IVIg induced increase in cellularity of DLNs as well as antigen specific increase in proliferation and cytokine/chemokine expression in

DLNs that may be due to down-regulated S1P1 mediated egress of CD4 cells. As well as an increase in the frequency of Th1 cells and the expression of the Th1 chemokine CCL5 that may be the result of altered immune synapse functioning during APC presentation. This chemokine could be partially responsible for increased leukocyte infiltration of the anterior CNS.

### Future Directions and Potential Limitations

Given our results there are several directions in which this research should proceed in order to further understand the mechanisms involved with IVIg in this model of EAE. Although we did not see a modulation in the CD4 populations as we hypothesized, IVIg may still be modulating the disease in this model via effects on integrin mediated migration of leukocytes in the brain. A future study looking at the composition of the infiltrating CD4 cells of the cerebellum, brain stem and ventricles should conclusively determine if the non-classical phenotype in the PLP<sub>139-151</sub> EAE model is due to a modulation of Th1/Th17 as has been reported in various other EAE models.

We did not originally perform those studies due to the technical challenge of isolating leukocytes from the lower brain structures. One reason is that the amount of myelin in relation to the number of mononuclear cells infiltrating (especially at the early time point) is much greater than in the spinal cord and many infiltrating leukocytes are lost in the debris during the process of isolation. It remains possible that if we examined the cerebellum and areas surrounding the choroid plexus and ventricles, especially at the earlier time point, we might have been able to see a shift in the ratio of Th1 and Th17

populations infiltrating the CNS. This would be done most effectively by immunohistochemical staining of brain sections specifically examining markers for Th1 and Th17 CD4<sup>+</sup> cells.

In light of this data and recently reported effects of VLA-4 on B cell migration and activation, the effects of IVIg on B cell differentiation and migration should be examined in detail in this model. In particular, identifying the phenotype(s) of the depressed B cell population from the spinal cords of non-classical EAE mice as well as the spleens where it has been shown that early Breg proliferation occurs would help to understand this feature of our model. Using the described markers for Bregs should make differentiating these B cell populations in the CNS and periphery relatively straightforward. If proven to be Bregs, future studies could focus on isolating this cell population and demonstrating Breg effector function either *in vitro* co-culture studies or adoptive transfer into IVIg treated mice to show amelioration of the non-classical phenotype. This would be highly challenging since the overall B cell numbers are quite small and isolating enough cells to experiment with would be difficult. Another possibility would be to use B cell depletion by anti-CD20 to see if we observe the same non-classical disease phenotype and pattern of leukocyte infiltration of the CNS. Additionally a comparison of IVIg treatment to anti-VLA-4 treatment in this model could be informative.

A closer examination of CD8 cells associated with the anterior spinal cord may reveal a new aspect of the PLP model not previously described. If indeed the infiltrating CD8 population can be shown to be CTLs and that they are mediating the damage we observe in the anterior spinal cord, this would have implications for how PLP<sub>139-151</sub> is recognized and induces disease in this model. It would be the first demonstration of



PLP<sub>139-151</sub> inducing a CD8<sup>+</sup> population that is associated with CNS pathology. Closer examination by histology would reveal if these cells were associated with demyelinated lesions and were degranulating, releasing their cytotoxic mediators, and thus acting as cytotoxic CD8 cells in our model. This would help to explain the demyelination and non-classical disease phenotype we observe given their location in the anterior spinal cord. If we found these cells expressing IL-10 by intracellular staining, then we might discount them as CTLs. However given the demonstrated plasticity of CTLs and CD8 Tregs, this might not be straightforward by flow cytometry (Sun, Whitaker et al. 2001, Ortega, Kashi et al. 2013).

Additionally our observations made in the DLNs in this model should be further investigated. In particular, whether our observations are due to IVIg-mediated inhibition of migration of leukocytes from secondary lymphoid tissue or if there are direct effects on activation, proliferation and polarization of various lymphocyte populations in the DLNs. It remains a possibility that both are occurring in this model. One way to see if our observations are due to an increase in DLN cellularity due to IVIg inhibiting migration or a direct influence of IVIg on T cell activation and proliferation would be to purify the CD4<sup>+</sup> cells from the DLNs. Once purified, CD4<sup>+</sup> cells would be stimulated in equal numbers from vehicle and IVIg treated mice. If the IVIg and vehicle treated cells proliferated equally, and secreted similar levels of cytokines, then the likely answer would be that egress from the DLN was inhibited by IVIg and that activation and polarization were not affected. However if IVIg-treated CD4<sup>+</sup> cells proliferated and secreted more cytokines, then it would suggest increased activation mediated by IVIg. Additionally if IVIg is behaving the way Thein and Glatigny observe with anti-VLA-4 in their EAE models, addition of anti-VLA-4 to our model might recapitulate our non-classical EAE observation with the concurrent increase in CD4<sup>+</sup> IFN $\gamma$ <sup>+</sup> cells in the DLNs

and an increase in CCL5 expression (Theien, Vanderlugt et al. 2001, Glatigny, Wagner et al. 2016).

One question that still remains is why IVIg in this model of EAE seems to function so differently than in other models of EAE, and especially the MOG models. As mentioned previously, pertussis toxin has immuno-modulatory and BBB activity that may affect normally migrating lymphocytes. Studies comparing MOG EAE with and without pertussis looking at IVIg effects on B cell and CD8 cell activity and function compared to the PLP<sub>139-151</sub> model might be instructive in better understanding what the differences between these models are.

### Clinical Implications for Human Disease

This research has implications for Multiple Sclerosis and especially current therapeutic strategies being investigated in the clinic. It is well appreciated that MS is a heterogeneous disease and that this heterogeneity may suggest multiple related diseases with differing underlying mechanisms (Lucchinetti, Bruck et al. 1996). If the observations of IVIg effects on B cell populations in this model and the phenotype we observe are confirmed and better defined, it could provide an avenue to better understand B cell functions in both the pathology and treatment of forms of the disease in which B cells are highly implicated.

Natalizumab (anti-VLA-4 mAb) is currently being studied in clinics for its activity on pathogenic B cells in RR and SP MS patients (Hausler, Nessler et al. 2015,

Warnke, Stettner et al. 2015, Lehmann-Horn, Sagan et al. 2016). Natalizumab has shown great promise for relief in SP MS and PP MS however there still remains the possibility of lethal complications such as PML with its use. If IVIg is in fact acting through modulation of VLA-4/selectin interactions with B cells instead of Th1 cells as we previously hypothesized and if it can be demonstrated that the effects we observe on B cell migration apply to pathogenic B cells as well as regulatory B cells then it might be possible to tailor IVIg for patients that have B cell mediated forms of disease. In addition since IVIg is well tolerated and has few side effects, it may be possible to tailor it for use in conjunction with natalizumab to increase potency while reducing chances of the direct side effects of anti-integrin therapy.

Another biologic that has received quite a bit of research in the past decade for the treatment of MS is the B cell depleting monoclonal Ab, rituximab. This antibody directed at CD20 leads to rapid depletion of circulating B cells and is associated with marked improvement in symptoms for some MS patients, especially those with B cell associated SP forms of disease (Topping, Dobson et al. 2016). It has been proposed that pro-inflammatory myelin reactive memory B cells are the populations that are most implicated with the inflammation associated with B cells in MS. These cells are very potent APCs including in the CNS and may serve to reactivate pathogenic T cells and exacerbate pro-inflammatory functions of other leukocytes. It is not clear the degree that the myelin reactive antibodies they produce are responsible for inflammation and lesion formation in the CNS since clinical benefits are observed soon after cellular depletion and before auto-antibody levels decrease in circulation of MS patients. This would imply that the pathogenic effector functions of these cells are due to their APC activity or the pro-inflammatory profile of the cytokines/chemokines they express (von Budingen, Palanichamy et al. 2015). If it can be demonstrated that IVIg in our model is mediating

some of its activities through modulation of APC presentation (including B cells) then this model may serve to better understand the activities of B cell depletion therapy as well. In addition just as with natalizumab, IVIg may be valuable if tailored correctly as an adjunct therapy to rituximab.

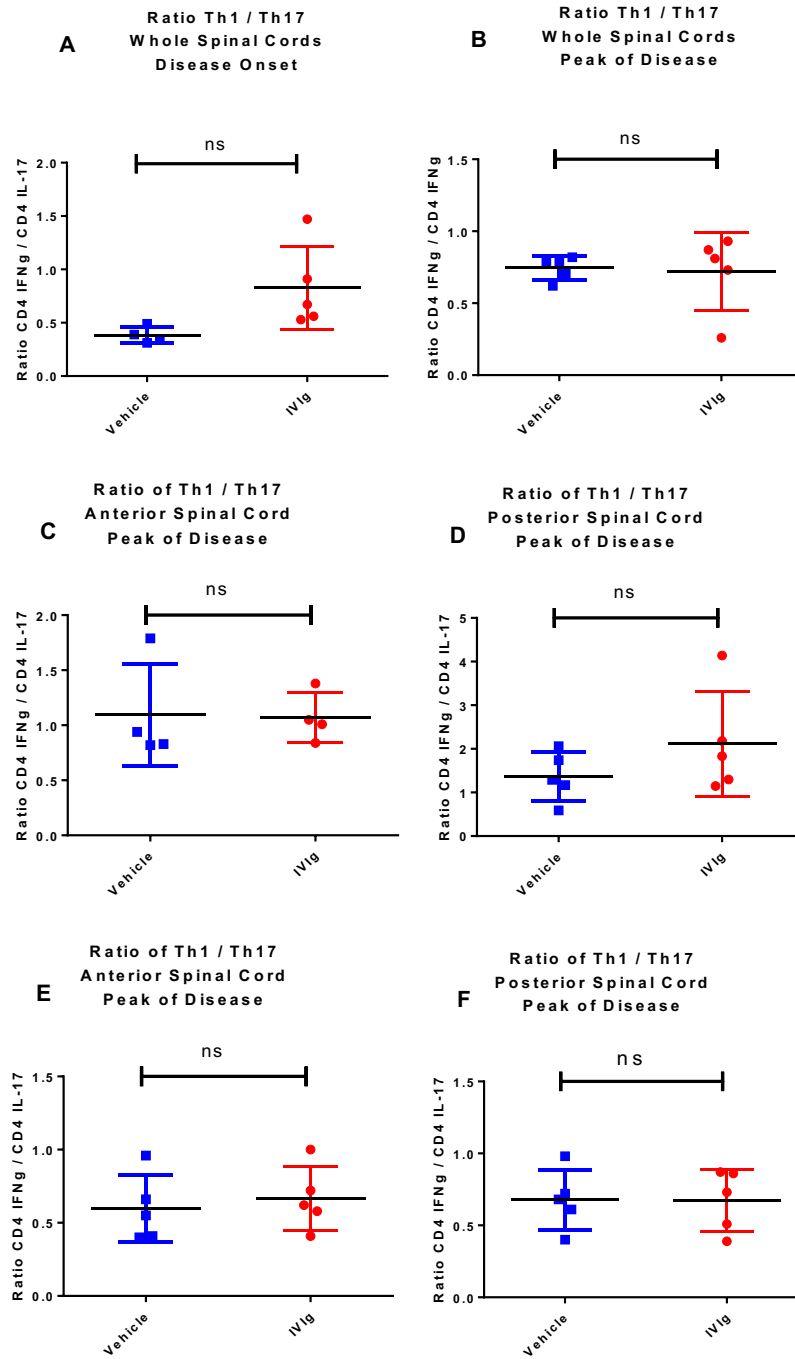
Finally this model of EAE itself could have implications for MS research. Much research has been directed towards understanding the reasons for brain vs spinal cord inflammation in EAE and MS. This has direct implications for understanding the differences between the model and human disease. As described earlier inflammation in EAE is generally localized to the spinal cord while in MS inflammation is initially localized to the brain. If the effects of IVIg in this model of EAE are better characterized, it could potentially serve as a useful model to better understand the differences between EAE and MS. In addition it may shed light on the differences of RR MS and the more progressive forms of the disease.

## Conclusions

Although we were unable to prove our original hypothesis and also to identify a single unifying principle for our observations, the groundwork has been laid and provides a solid direction in which to move this research forward. Given that IVIg is well known to have pleiotropic activity and to function in multiple capacities, being unable to identify a single mechanism or cell population to explain our non-classical EAE observations is not unexpected. However our observations in this model potentially implicate several interrelated biological processes. In addition this model may shed light on some of the

key differences between EAE and MS. By teasing out these observations it should be possible to gain a better understanding of how this therapeutic works and potentially how it could be targeted more precisely for various disease applications. In particular if B cells are affected in this model the way we suspect, this could provide a tool for investigating forms of MS that have B cell dysregulation and brain inflammation as major features as well as gain a better understanding of causes of RR versus progressive forms of the disease.

## Appendix 1 Th1/Th17 ratios for spinal cords



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